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(54) Title: USE OF ANTI-VEGF ANTIBODY TO ENHANCE RADIATION IN CANCER THERAPY

(57) Abstract

We have discovered that VEGF expression is induced following exposure tumors to ionizing radiation (IR) both *in vitro* and *in vivo*. We found that treatment of tumor-bearing mice with a neutralizing antibody to VEGF prior to irradiation is associated with greater than additive antitumor effects.

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USE OF ANTI-VEGF ANTIBODY TO ENHANCE RADIATION IN CNACER THERAPY

BACKGROUND

This work was supported by grants from the National Cancer Institute, and the government have certain rights in the invention.

Tumors influence the surrounding host stroma by inducing angiogenesis to supply their oxygen and nutrient needs, allowing them to grow. In normal tissues, angiogenesis is tightly regulated by the balance between angiogenic and anti-angiogenic factors[1,2]. However, the induction of angiogenesis by tumor-derived pro-angiogenic proteins is a discrete component of the malignant phenotype. Decreased production of angiogenesis inhibitors or increased expression of angiogenic peptides can shift the balance towards a pro-angiogenic state[2], permitting tumor growth. As a tumor increases in size, it disrupts its surrounding stroma and recruits still more host blood vessels. This paracrine relationship between a tumor and its blood supply represents a potential point of attack for antitumor therapy.

A family of angiogenic peptides, isoforms of vascular endothelial cell growth factor (VEGF), are expressed by many human tumors and normal cells[3-5]. VEGF is the only known angiogenic protein that is exclusively mitogenic for endothelial cells *in vitro* and strongly angiogenic *in vivo*[4,5]. It is secreted by a wide variety of human tumors, and inhibition of VEGF-induced angiogenesis, either by neutralizing antibodies or a dominant negative soluble receptor, blocks the growth of primary and metastatic experimental tumors[6-8]. Physiologic regulators of VEGF expression include hypoxia[9,10] and cytokines[3,5]. In certain human tumors, oncogenic mutations of *ras* and p53 are associated with increases in intratumoral VEGF levels and a poor prognosis[11,12].

SUMMARY OF THE INVENTION

In the present study, we examined the production of VEGF by LLC and human xenograft tumors following exposure to ionizing radiation (IR), and found that VEGF expression is induced following IR. We next examined whether blocking the action of a positive regulator of

angiogenesis could potentiate the antitumor effect of IR.Blocking this IR-mediated increase in VEGF using neutralizing antibodies against VEGF resulted in increased endothelial cell killing and produced greater than additive anti-tumor effects in mouse tumor model systems, findings that support a model in which induction of VEGF by IR contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity.

The invention provides a method of reducing tumor radio resistance or chemotherapy resistance in a cancer patient being or to be treated with radiation or chemotherapy, by administering to the patient a substance that inhibits chemotherapy or radiation-induced VEGF expression or that blocks VEGF activity in the patient.

The substance can be an anti-VEGF antibody, and can be administered (preferably IV) shortly (1-4 hours) prior to chemotherapy or radiation treatment.

The dosages, timing, and duration of anti-VEGF antibody administration in humans can be extrapolated from the animal model results presented herein. Antibody preferably is administered intravenously, either prior to, during, or following radiation or chemotherapy administration.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. VEGF levels in Lewis lung carcinoma in vivo and in vitro. ***A. VEGF mRNA levels in LLC tumors following IR exposure (40 Gy divided into two daily doses).*** Total RNA was isolated from representative tumors and probed with a cDNA encoding human VEGF-165, after which they were stripped of probe and reprobed with a cDNA to GAPDH to demonstrate message integrity. By scanning densitometry, normalized to GAPDH, VEGF mRNA is induced 3-fold following IR exposure. Blots from representative animals are displayed. ***B. VEGF protein levels in media conditioned by LLCs following IR exposure.*** LLCs were plated in six-well plates at low density (25% confluence), allowed to attach overnight, and then irradiated with 0, 5, 10, or 20 Gy. Conditioned media was collected every 24 hrs, and cells were detached with trypsin and counted. VEGF levels were normalized to the number of cells and reported as total pg VEGF/10^6 cells. No VEGF was detectable in unconditioned media.

Figure 2. VEGF expression in human tumor cell lines. Subconfluent cells from human tumor cell lines (Seg-1 esophageal adenocarcinoma, SQ20B squamous cell carcinoma, U1 melanoma, and U87 and T98 glioblastoma) were exposed to 10 Gy of ionizing radiation. Conditioned media from radiated and unirradiated cells was collected 24 hours later. VEGF levels in conditioned media were measured by ELISA and normalized to cell number. An increase in VEGF secretion was observed in each cell line: Seg-1 (p=?), SQ20B (p=0.08), T98 (p=0.02), U1 (p=0.009), U87 (p=0.0009). No VEGF was detectable in unconditioned media.

FIGURE 3. Effect of VEGF blockade prior to treatment with ionizing radiation in mouse tumors and human xenografts. LLC cells (1×10^6) were injected subcutaneously into the hindlimbs of female C57Bl/6 mice. SQ20B squamous cell carcinoma cells (5×10^6) and Seg-1 esophageal adenocarcinoma cells (3×10^6) were injected into the hindlimbs of female

athymic nude mice. Tumors were allowed to attain a mean size between 350-450 mm^3 (LLC, 442 ± 14 mm^3; SQ20B, 372 ± 16 mm^3; Seg-1, 407 ± 20 mm^3), after which treatment was begun. *A. Effect of VEGF blockade prior to ionizing radiation in LLC tumors.* Mice were treated as follows: IR, 40 Gy administered as two 20 Gy doses on days 0 and 1; IR (40 Gy) plus polyclonal goat anti-mouse VEGF-164 antibody. 10 µg were administered intraperitoneally 16 and 3 hrs before the first IR treatment and 3 hours before the second IR treatment (3 doses total); goat anti-mouse VEGF-164 antibody alone administered as described. Untreated controls received nonimmune goat IgG. ***B.*** *Effect of VEGF blockade prior to ionizing radiation in SQ20B xenografts.* Mice were treated as follows: IR, 40 Gy administered as four 10 Gy doses on days 0, 1,2, and 3; IR (40 Gy) plus monoclonal anti-human VEGF-165 antibody, 10 µg administered intraperitoneally two to three hours before each dose of IR; monoclonal anti-human VEGF-165 antibody alone administered identically to the combined treatment group. Untreated controls received nonimmune mouse IgG. *C. Effect of VEGF blockade prior to ionizing radiation in Seg-1.* Mice were treated as follows: IR, 20 Gy administered as 4-5 Gy doses on days 0, 1,2, and 3; IR (20 Gy) plus monoclonal anti-human VEGF-165 antibody, 10 µg administered intraperitoneally two to three hours before each dose of IR; monoclonal anti-human VEGF-165 antibody alone administered identically to the combined treatment group. Untreated controls received nonimmune mouse IgG. *D. Mice bearing SQ20B xenografts from different treatment groups (day 22).* Mice with tumor volumes closest to the mean for each group were chosen.

FIGURE 4. Effect of manipulating VEGF levels in vitro on IR-mediated vascular endothelial cell killing. For MTT assays, HUVECs were plated in 96-well plates at 1 x 103 cells/well and treated with either differing concentrations of recombinant human VEGF-165 or monoclonal anti-human VEGF-165 antibody prior to treatment with IR, and absorbance readings

measured at varying time points after IR (see Methods). For clonogenic survival assays, HUVECs were treated with different concentrations of VEGF or a polyclonal goat anti-VEGF-165 antibody four hours prior to irradiation (see Methods). *A. MTT assay for HUVECs pretreated with varying concentrations of recombinant human VEGF-165 four hours before IR treatment. Absorbance measurements were taken at 96 hrs after IR and normalized to those obtained under standard conditions (no IR treatment and VEGF=10 ng/ml). B. Clonogenic survival assay for HUVECs pretreated with 1, 10, or 50 ng/ml VEGF ± 200 and 900 cGy. Surviving fraction is normalized to plating efficiency for unirradiated cells. C. MTT assay for HUVECs pretreated with monoclonal anti-VEGF-165 antibody four hours before IR treatment. Absorbance measurements were taken at 96 hrs after IR and normalized to those obtained with no pretreatment with Ab. VEGF=10 ng/ml for this experiment. D. Clonogenic survival assay for HUVECs pretreated with a monoclonal anti-VEGF-165 antibody prior to irradiation.*

DETAILED DESCRIPTION

Ionizing radiation induces tumor VEGF production in vivo and in vitro

We have examined the production of VEGF in Lewis lung carcinoma (LLC) tumors following exposure to ionizing radiation (IR). LLC cells (1×10^6) were injected subcutaneously in the hindlimbs of female C57BL/6 mice and allowed to grow to a volume of 510 ± 11 mm^3 (approximately 2.5% body weight). Tumors were irradiated with 20 Gy on days 0 and 1 and then harvested at days 2, 5, or 14. VEGF levels were measured by ELISA and normalized to total tumor protein. VEGF levels in extracts from control tumors remained relatively constant (46 to 90 pg/mg total protein) for 14 days as the tumors grew to 6110 ± 582 mm^3, or approximately 30% of body weight (Table 1). By contrast, on day 2, the mean VEGF level in irradiated tumors was increased more than three-fold as compared to that in unirradiated tumors (234 ± 79 pg/mg total extract protein, p=0.032). The mean VEGF level in irradiated tumors remained 2.2-fold higher than that in unirradiated tumors at day 14 (194 ± 47 pg/mg total extract protein, p=0.027). Plasma VEGF levels remained low or undetectable in control and irradiated animals (data not shown). To confirm the effects of IR, VEGF mRNA levels were assessed in the same tumors by Northern blot analysis. VEGF transcripts were induced 3-fold two days after exposure to IR

(Figure 1A). Moreover, VEGF mRNA levels remained elevated for fourteen days. These findings demonstrate that IR induces VEGF expression *in vivo*.

To determine whether IR induces VEGF in tumor cells *in vitro*, subconfluent LLC cells were exposed to different doses of IR, and conditioned media was harvested at various intervals for measurement of VEGF levels by ELISA. VEGF levels in LLC-conditioned media exhibited an IR dose-dependent increase within 24 hours. At 72 hours, VEGF levels were nearly 6-fold higher in media from LLC irradiated with 20 Gy (Figure 1B), as compared to that for control cells ($p=0.009$). VEGF expression was also studied in irradiated human tumor cell lines: Seg-1 (esophageal adenocarcinoma)[13]; SQ20B (a radioresistant squamous cell carcinoma line)[14]; U1 (melanoma); and T98 and U87 (glioblastoma). Under basal conditions, these tumor cell lines secreted widely differing levels of VEGF, with U87 cells producing the most VEGF and U1 melanoma cells the least (Figure 2). All demonstrated an IR-dependent increase in VEGF production within 24 hours of treatment with 10 Gy (Figure 2). These findings demonstrate that IR induces VEGF expression in diverse tumor cell types.

Blocking VEGF action enhances the in vivo antitumor effect of ionizing radiation

To determine whether induction of VEGF secretion by tumors affects anti-tumor response, we treated LLC tumors with neutralizing antibodies to VEGF prior to IR exposure. Female C57BL/6 mice bearing LLC tumors (559 ± 51 mm^3) were treated with a polyclonal goat antibody directed against recombinant murine VEGF-164 (R & D Systems, 10 µg qd by intraperitoneal injection) or with nonimmune goat IgG. By day 7, tumors from control animals had attained a volume of 2713 ± 346 mm^3, while tumors in anti-VEGF-treated mice were 1011 ± 266 mm^3 ($p=0.02$). Consistent with previous observations[6,15-17], these findings indicate that blocking VEGF activity inhibits tumor growth. To evaluate the antitumor effects of combining

anti-VEGF antibodies and IR, mice bearing LLC tumors were treated as follows: untreated control; IR alone, 20 Gy on consecutive days (40 Gy total); anti-VEGF antibody; and IR plus anti-VEGF antibody (Figure 3A). Starting from a mean volume of 442 ± 14 mm^3 at day 0, tumors in untreated controls reached a mean volume of 1389 ± 136 mm^3 by day 6. Treatment with anti-VEGF antibody alone produced a 42.6% reduction in tumor volume (796 ± 41 mm^3, p=0.004); IR alone, 43.0% reduction (792 ± 30 mm^3, p=0.006); and the combination of IR and anti-VEGF antibody, 78.0% reduction (305 ± 58 mm^3, p=0.001 relative to IR alone), a greater than additive effect (Table II).

To extend these findings to other models for tumors, we examined the effect of combining anti-VEGF antibody with IR in human squamous cell carcinoma and esophageal adenocarcinoma xenografts, both of which represent human tumors for which IR is a major therapeutic modality. First, athymic nude mice bearing radioresistant human head and neck squamous cell carcinoma xenografts (SQ20B)[14] were treated with IR and a neutralizing monoclonal antibody against human VEGF-165 (R & D Systems, Inc.). SQ20B cells (5×10^6) were implanted in the hindlimbs of female athymic nude mice and allowed to attain a volume of 372 ± 16 mm^3 (Figure 3B), after which they were treated with IR alone (40 Gy given as four 10 Gy fractions), anti-VEGF antibody alone (10 µg intraperitoneally each day for four doses), or combined IR and anti-VEGF antibody (10 µg antibody administered 3 hours prior to treatment with IR). On day 19, tumors in untreated controls reached a mean volume of 3671 ± 790 mm^3. Treatment with anti-VEGF antibody alone produced a 28.5% reduction in mean tumor volume (2624 ± 287 mm^3); IR alone, a 48.8% reduction (1793 ± 279 mm^3); and the combination of IR and anti-VEGF antibody, a 81.8% reduction (669 ± 120 mm^3, p=0.003 relative to IR alone). Next, we examined a xenograft model for a human cancer that is seldom cured by IR alone,

esophageal adenocarcinoma. Seg-1 cells[13] (3×10^6) were implanted in the hindlimbs of athymic nude mice and allowed to attain a volume of 407 ± 20 mm^3 (Figure 3C), after which they were treated with IR alone (20 Gy in daily 5 Gy fractions), anti-VEGF antibody as above, or combined therapy. Similar enhancement of the antitumor effect of IR by anti-VEGF antibody was observed. As was the case for LLC, in both human xenografts, the anti-tumor effects of combined therapy were greater than additive (Table II). These findings suggest that blocking the effects of VEGF enhances the tumoricidal effects of IR.

Blocking VEGF increases endothelial cell killing by ionizing radiation

To assess the potential effects of VEGF on IR-mediated killing of tumor cells and endothelial cells, we measured *in vitro* survival of LLCs and human umbilical vein endothelial cells (HUVECs) following exposure to IR. By MTT assay, there was no detectable cytotoxicity of LLC or SQ20B cells following exposure to VEGF or anti-VEGF antibody (data not shown). In addition, recombinant VEGF failed to protect LLC or SQ20B cells from IR-mediated killing (data not shown), and there was no interactive cytotoxicity of LLC when anti-VEGF antibody was combined with IR (data not shown). Next, the effect of exogenous VEGF protein on IR-mediated cell killing of HUVECs was assessed by MTT[18] and clonogenic assays[19] (Figure 4). As measured by the MTT assay 96 hours after IR, pretreatment with VEGF protected HUVECs against the cytotoxic effects of 10 Gy IR in a dose-dependent fashion (Figure 4A-NEED p VALUES). This effect was observed both in the presence and absence of serum (data not shown). Clonogenic survival was also increased in a dose-dependent fashion when VEGF was added to the HUVEC culture medium prior to IR (Figure 4B). Next, we tested whether adding anti-VEGF increases endothelial cell sensitivity to IR. Adding anti-VEGF antibody to the culture medium prior to IR exposure decreased cell proliferation as measured by MTT assay in

HUVECs but not SQ20B cells (Figure 4C) or LLC proliferation (data not shown). Similar decreases in endothelial cell survival after exposure to IR were observed in clonogenic assays when HUVECs were pretreated with anti-VEGF antibody (Figure 4D). To determine whether VEGF protects against IR-induced apoptosis, flow cytometry studies were performed after labeling cells with 7-AAD[20]. There was no difference between the percentage of apoptotic cells in HUVECs exposed to IR, concentrations of anti-VEGF monoclonal antibody as high as 100 ng/ml, or both IR and anti-VEGF antibody (DATA NEEDS TO BE FINALIZED). These results indicate that IR-induced VEGF production by tumors inhibits the lethal effects of IR on endothelial cells.

DISCUSSION

Our findings demonstrate that IR induces VEGF expression by tumors. Importantly, blocking the effect of VEGF in irradiated LLC and human tumors produces greater than additive antitumor effects *In vivo*. Also, blocking VEGF action produces increased clonogenic killing of vascular endothelial cells *in vitro*, whereas the addition of recombinant VEGF blocks the killing of endothelial cells. Taken together, these data raise the possibility that blocking positive regulators of angiogenesis is effective in potentiating the antitumor effects of IR. The use of growth blockade for endothelial cells (anti-VEGF antibody) and IR may disrupt the paracrine relationship between the tumor and its blood supply and emphasizes the potential importance of combining an angiogenesis inhibitor with a DNA damaging agent. IR is a major therapeutic modality that is effective in the treatment of relatively small tumors and of large tumors only with considerable toxicity to normal tissues. Depriving the tumor endothelium of VEGF using neutralizing antibodies prior to IR exposure or pretreating tumor vessels with antiangiogenic

peptides represent strategies to increase the anti-tumor effects of IR with minimal toxicity to normal tissues.

METHODS

Cell culture

Lewis lung carcinoma cells (gift of J. Folkman) and SQ20B cells were grown as previously described[19,21,22]. Human umbilical vein endothelial cells (HUVECs) were maintained in EGM-2 medium (Clonetics) + 1% fetal bovine serum (Clonetics). U87 and T98 human glioblastoma cells were maintained in RPMI-1640 (Life Technologies, Inc.) + 10% FBS (Intergen); U1 melanoma cells, DMEM (75%) + F12 (25%) + 10% FBS.

Neutralizing antibodies against VEGF

For experiments with LLC, neutralizing polyclonal goat antibody (IgG) against recombinant mouse VEGF-164 (R & D Systems, Inc.) was dissolved in PBS and administered via intraperitoneal injection. Control mice in these experiments received nonimmune goat IgG (Sigma). For experiments with human tumor xenografts, a neutralizing monoclonal antibody to recombinant human VEGF-165 (R & D Systems) was used. Control mice in these experiments received nonimmune mouse IgG (Sigma).

Tumor models

LLC cells were injected subcutaneously into the right hind limb (1×10^6 cells in PBS) of C57BL/6 female mice (Frederick Cancer Research Institute). SQ20B human squamous cell carcinoma cells[14] (1×10^6 cells) and Seg-1 esophageal adenocarcinoma cells[13] (3×10^6 cells) were injected subcutaneously into the hind limb of female athymic nude mice (Frederick Cancer

Research Institute). Tumor volume was determined by direct measurement with calipers and calculated by the formula (length x width x depth/2) and reported as the mean volume ± s.e.m., as previously described[19,21]. Tumors were allowed to grow to a volume of 300-500 mm^3, at which time mice were divided into experimental groups and treatment begun. Tumors were irradiated using a GE Maxitron X-ray generator operating at 150 kV, 30 mA, using a 1 mm aluminum filter at a dose rate of 188 cGy/min.. Mice were shielded with lead except for the tumor-bearing right hindlimb. The care and treatment of animals was in accordance with institutional guidelines.

Measurement of VEGF levels in tumor extracts and conditioned media

At various time points, mice were chosen from each LLC experimental group such that the overall group mean tumor volume was affected as little as possible and euthanized to obtain tumor tissue. Tumor extracts were prepared by homogenizing tumors in RIPA buffer (150 mM NaCl, 10 mM Tris, 5 mM EDTA, Triton X-100 0.5%, and dithiothreitol 1 µM, pH 7.5, PMSF 50 µM, leupeptin 1 µg/ml, and aprotinin 2 µg/ml). The homogenate was then subjected to three freeze-thaw cycles in liquid nitrogen to lyse cells and then spun at 5000 G at 4° C to pellet debris. VEGF levels were measured in tumor extract supernatants by ELISA (R & D Systems), and protein assays were performed by Lowry assay. VEGF levels were normalized to total extract protein concentration and expressed as pg VEGF/mg total extract protein. VEGF levels in tumor cell conditioned media were also measured by ELISA and were normalized to cell number in each well. At least three wells per time point were measured.

$$P = \frac{A - A_0}{A_{control} - A_0}$$

where P= proliferation relative to control; A= absorbance at 515 nm (A_{515}); A_0= A_{515} at T=0 hr; and $A_{control}$=A_{515} for control cells (unirradiated, grown in 10 ng/ml VEGF-165).

Clonogenic assays

Clonogenic assays were performed as previously described[19]. Briefly, HUVECs and LLCs were plated in EGM-2 media. Eighteen hours after plating, HUVEC media was replaced with media in which the VEGF supplied by the manufacturer was omitted, and a defined amount (0-50 ng/ml) of recombinant VEGF-165 (R & D Systems, Inc.) had been added. Four hours later, cells were irradiated with doses of 0-900 cGy using a GE Maxitron X-ray generator operating at 250 kV, 26 mA, with a 0.5 mm copper filter at a dose rate of 118 cGy/min. Cultures were returned to the incubator for 14-17 days, after which they were stained with crystal violet. Colonies were counted and surviving fractions were determined. Colonies containing >50 cells were scored as positive. For studies with antibodies, HUVECs were plated in serum-free EGM-2 containing 5 ng/ml VEGF-165. Four hours before irradiation, polyclonal antibodies to human VEGF-165 (R & D Systems, Inc.) were added to the media. Media was replaced with serum-containing media 48 hours after IR and the cells incubated for colony counting.

Data analysis

Statistical significance was determined using one-way analysis of variance (ANOVA) or Student's t-test, as appropriate.

TABLES AND TABLE LEGENDS

TABLE I: VEGF Levels in Lewis lung carcinoma tumors after irradiation

	UNTREATED CONTROLS		IONIZING RADIATION (40 Gy)	
Day	Mean tumor volume (mm^3) ± s.e.m	VEGF (pg VEGF/mg total protein)	Mean tumor volume (mm^3) ± s.e.m.	VEGF (pg VEGF/mg total protein)
2	947 ± 43	69 ± 21	641 ± 22	234 ± 79 *
5	1545 ± 93	46 ± 18	786 ± 52	135 ± 32 *
14	6110 ± 582	90 ± 23	2854 ± 338	194 ± 47 *

*$p < 0.05$ relative to VEGF levels untreated controls

TABLE II: Effect of combining anti-VEGF antibody and ionizing radiation

Tumor volume (% untreated control volume for untreated controls)

Tumor	Day	Ionizing radiation	Anti-VEGF antibody	Expected for combined if additive	Observed volume for combined	Observed/ expected
LLC	6	57.0%	57.4%	32.7%	*22.0%*	*0.673*
Seg-1	13	19.8%	77.8%	15.4%	*6.9%*	*0.448*
SQ20B	19	51.2%	71.5%	36.6%	*18.2%*	*0.497*

Northern blots

Total RNA was isolated from cultured cells and tumor tissue using the guanidine thiocyanate method[23] utilizing Trizol LS (Life Sciences, Inc.). 25 μg total RNA was fractionated on 1.2% agarose gels containing formaldehyde and blotted onto nylon membranes, then hybridized with a cDNA probe labeled by random priming and consisting of a cDNA encoding human VEGF[24]. Hybridizations were carried out at 60° C in 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate, 1 mM EDTA, and 1% bovine serum albumin[25], and blots were washed as previously described[26]. After autoradiography, blots were stripped of probe and rehybridized to a labeled cDNA encoding rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to demonstrate message integrity.

MTT Assays

HUVECs were plated (1×10^3 cells/well in 96 well plates) in EGM-2 media and allowed to attach overnight. Media was replaced with EGM-2 media containing different concentrations of recombinant human VEGF-165 (R & D Systems, Inc.). In other experiments, the concentration of VEGF-165 was kept constant and varying concentrations of either a neutralizing polyclonal or monoclonal anti-human VEGF-165 antibody (R & D Systems, Inc.) were added prior to treatment with IR. 72 or 96 hours after IR, cells were pulsed with 3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyltetrazolium bromide (Sigma Chemical Company)[18] at 0.5 mg/ml culture volume for four hours, after which the media was removed and the dye solubilized in dimethyl sulfoxide. Absorbance was measured at 515 nm and normalized to untreated control cells by the following equation:

REFERENCES

1. Hanahan, D., Christofori, G., Naik, P. & Arbeit, J. Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. *Eur. J. Cancer* 32A, 2386-2393 (1996).
2. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353-364 (1996).
3. Ferrara, N. & Davis-Smyth, T. The biology of vascular endothelial growth factor. *Endocr Rev* 18, 4-25 (1997).
4. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 13, 9-22 (1999).
5. Thomas, K.A. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J. Biol. Chem.* 271, 603-606 (1996).
6. Asano, M., Yukita, A., Matsumoto, T., Kondo, S. & Suzuki, H. Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor-121. *Cancer Res.* 55, 5296-5301 (1995).
7. Borgstrom, P., Hillan, K.J., Sriramarao, P. & Ferrara, N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer Res* 56, 4032-4039 (1996).
8. Goldman, C.K. *et al.* Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci USA* 95, 8795-8800 (1998).
9. Levy, A.P., Levy, N.S. & Goldberg, M.A. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 271, 2746-2753 (1996).

10. Minchenko, A., Bauer, T., Salceda, S. & Caro, J. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest* 71, 374-379 (1994).

11. Kieser, A., Weich, H.A., Brandner, G., Marme, D. & Kolch, W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 9, 963-969 (1994).

12. Rak, J. *et al.* Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 55, 4575-4580 (1995).

13. Hughes, S.J. *et al.* Fas/APO-1 (CD95) is not translocated to the cell membrane in esophageal adenocarcinoma. *Cancer Res* 57, 5571-5578 (1997).

14. Weichselbaum, R.R., Dahlberg, W. & Little, J.B. Inherently radioresistant cells exist in some human tumors. *Proc Natl Acad Sci USA* 82, 4732-4735 (1985).

15. Kanai, T. *et al.* Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. *Int J Cancer* 77, 933-936 (1998).

16. Yuan, F. *et al.* Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc Natl Acad Sci USA* 93, 14765-14770 (1996).

17. Asano, M. *et al.* Isolation and characterization of neutralizing monoclonal antibodies to human vascular endothelial growth factor/vascular permeability factor121 (VEGF/VPF121). *Hybridoma* 14, 475-480 (1995).

18. Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D. & Mitchell, J.B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer Res* 47, 943-946 (1987).

19. Mauceri, H. *et al.* Interaction of angiostatin and ionizing radiation in anti-tumour therapy. *Nature* 394, 287-291 (1998).

20. Philpott, N.J. *et al.* The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. *Blood* 87, 2244-2251 (1996).

21. Gorski, D.H. *et al.* Potentiation of the antitumor effect of ionizing radiation by brief concomitant exposures to angiostatin. *Cancer Res* 58, 5686-5689 (1998).

22. O'Reilly, M.S. *et al.* Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79, 315-328 (1994).

23. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* 162, 156-159 (1987).

24. Mukhopadhyay, D., Knebelmann, B., Cohen, H.T., Ananth, S. & Sukhatme, V.P. The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. *Mol Cell Biol* 17, 5629-5639 (1997).

25. Church, G.M. & Gilbert, W. Genomic sequencing. *Proc Natl Acad Sci USA* 81, 1991-1995 (1984).

26. Gorski, D.H. *et al.* Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G_0/G_1 transition in vascular smooth muscle cells. *Mol Cell Biol* 13, 3722-3733 (1993).

What is claimed:

1. Use of a substance that inhibits VEGF expression of blockers VEGF activity *in vivo* in the preparation of a medicament for the mediation of radio resistance or chemotherapy resistance in a human cancer patient.

2. The use of claim 1, wherein said substance is an anti-VEGF antibody.

FIGURES

FIGURE 1A

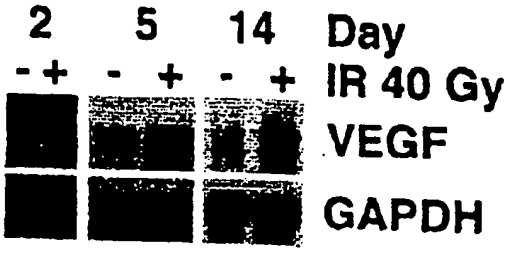


FIGURE 1B

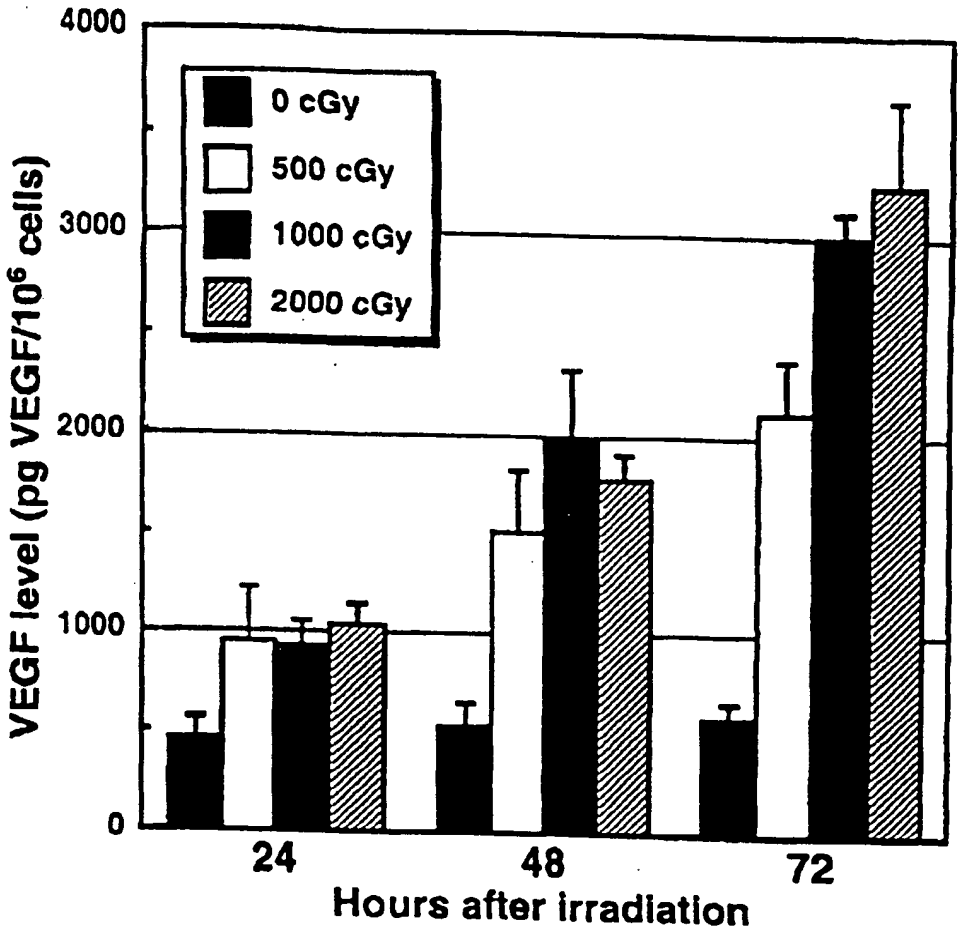
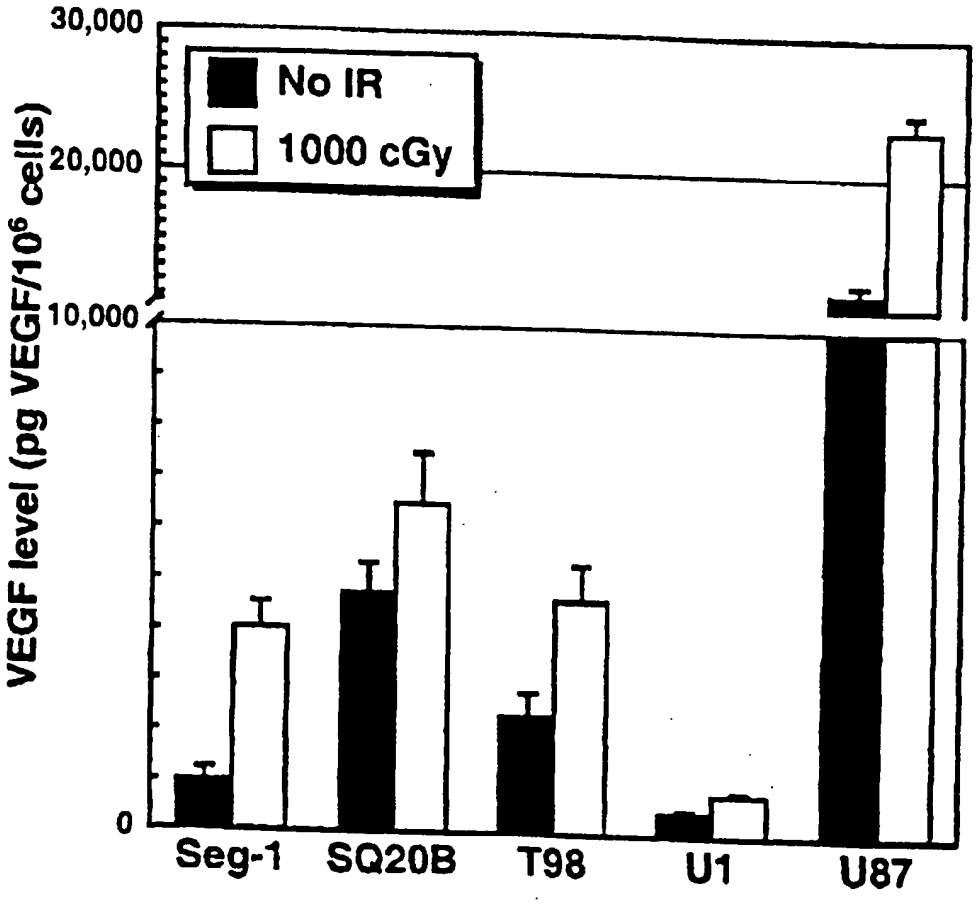

4000
3000
2000
1000
0
VEGF level (pg VEGF/10^6 cells)
0 cGy
500 cGy
1000 cGy
2000 cGy
24
48
72
Hours after irradiation

FIGURE 2



NOTE: Seg-1 DATA IS ONLY A PLACEHOLDER. REAL DATA IS PENDING

FIGURE 3A

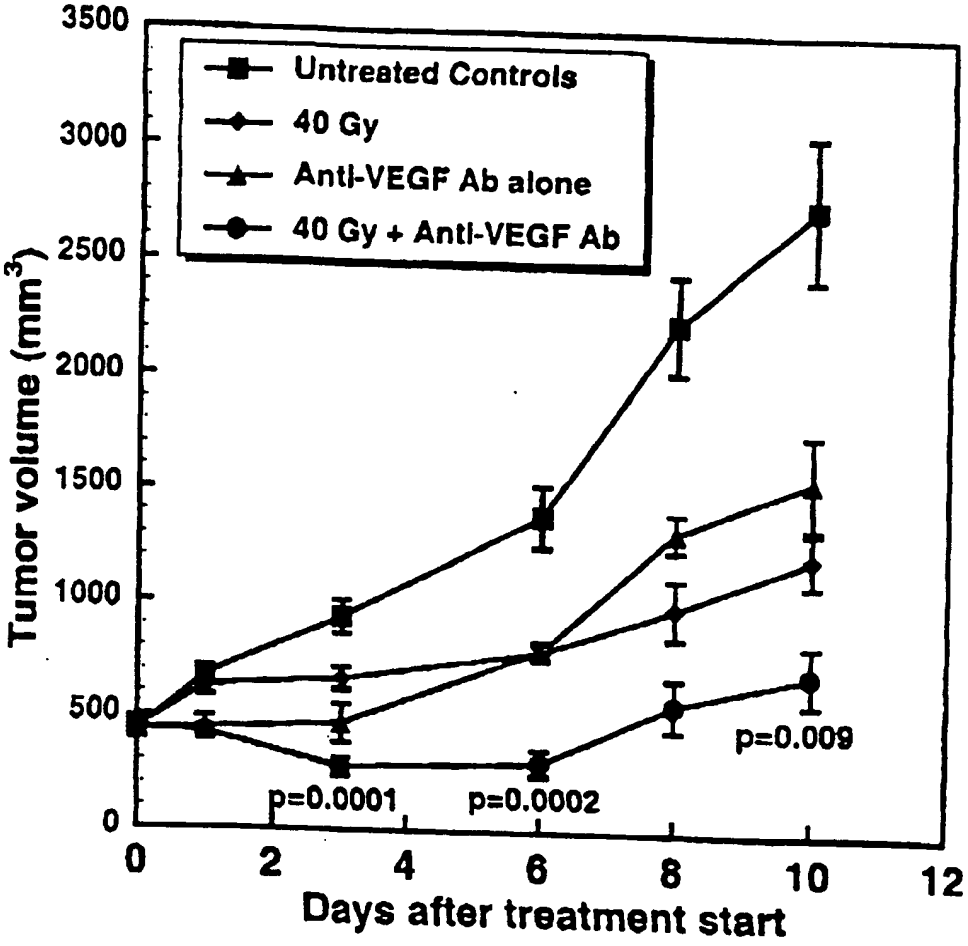

Untreated Controls
40 Gy
Anti-VEGF Ab alone
40 Gy + Anti-VEGF Ab
Tumor volume (mm3)
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
Days after treatment start
p=0.0001
p=0.0002
p=0.009

FIGURE 3B

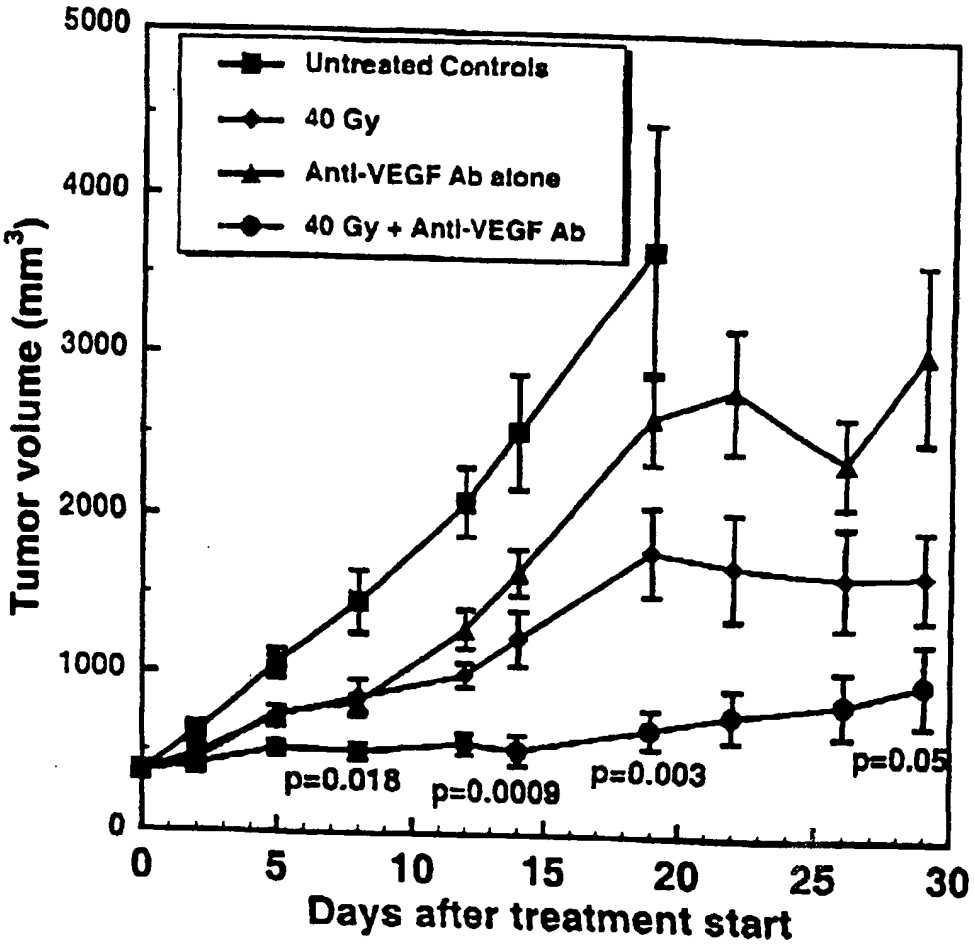

Untreated Controls
40 Gy
Anti-VEGF Ab alone
40 Gy + Anti-VEGF Ab
Tumor volume (mm3)
5000
4000
3000
2000
1000
0
p=0.018
p=0.0009
p=0.003
p=0.05
0
5
10
15
20
25
30
Days after treatment start

FIGURE 3C

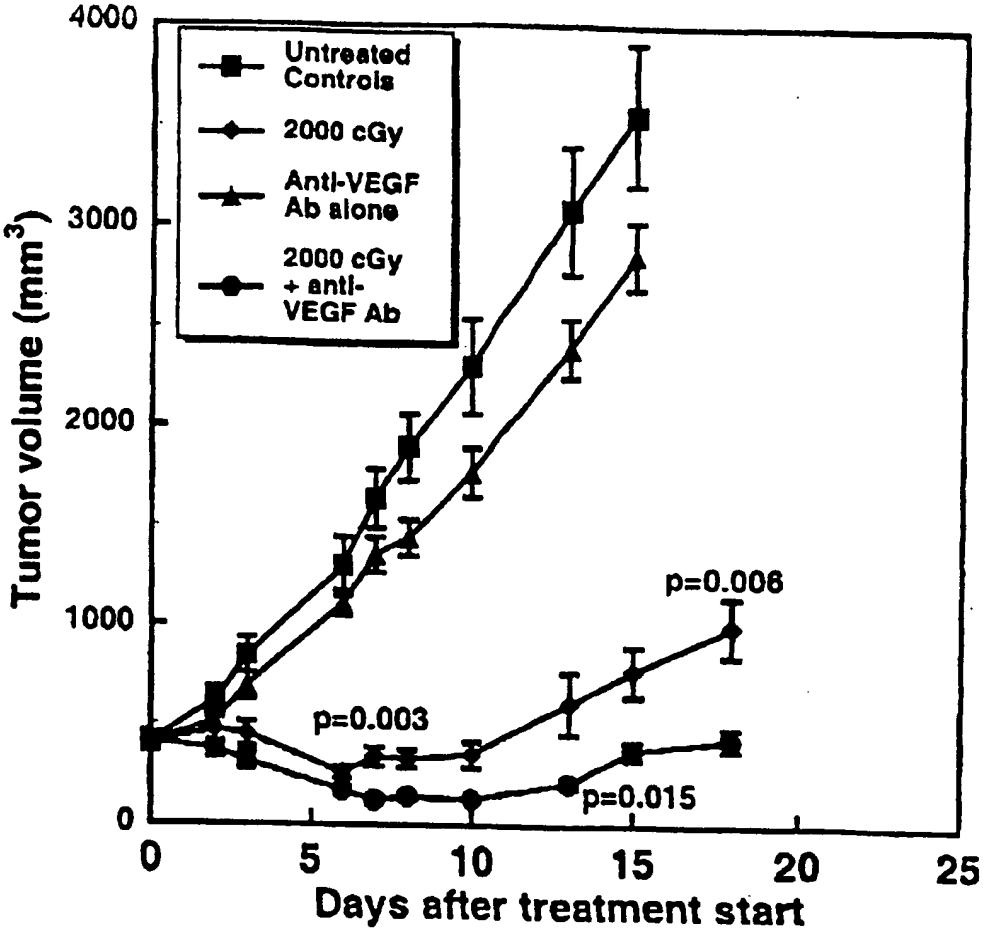

Untreated Controls
2000 cGy
Anti-VEGF Ab alone
2000 cGy + anti-VEGF Ab
Tumor volume (mm3)
4000
3000
2000
1000
0
0
5
10
15
20
25
Days after treatment start
p=0.006
p=0.003
p=0.015

FIGURE 3D

FIGURE 4A

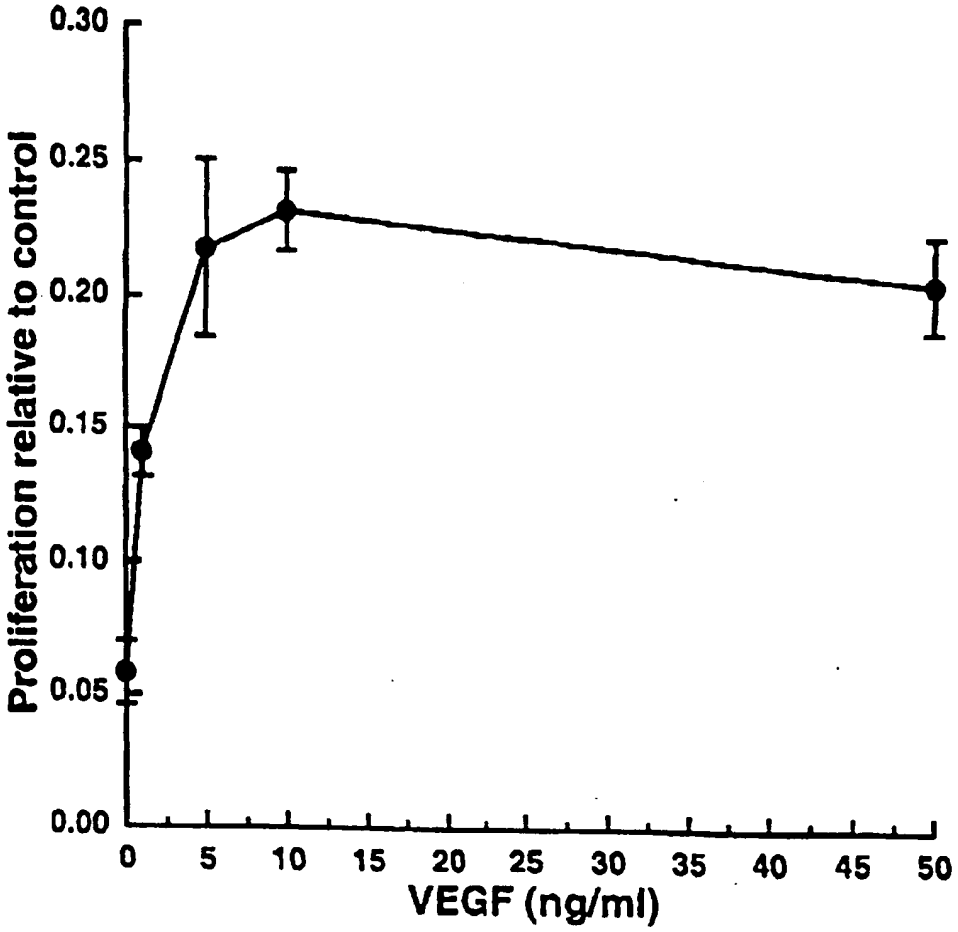
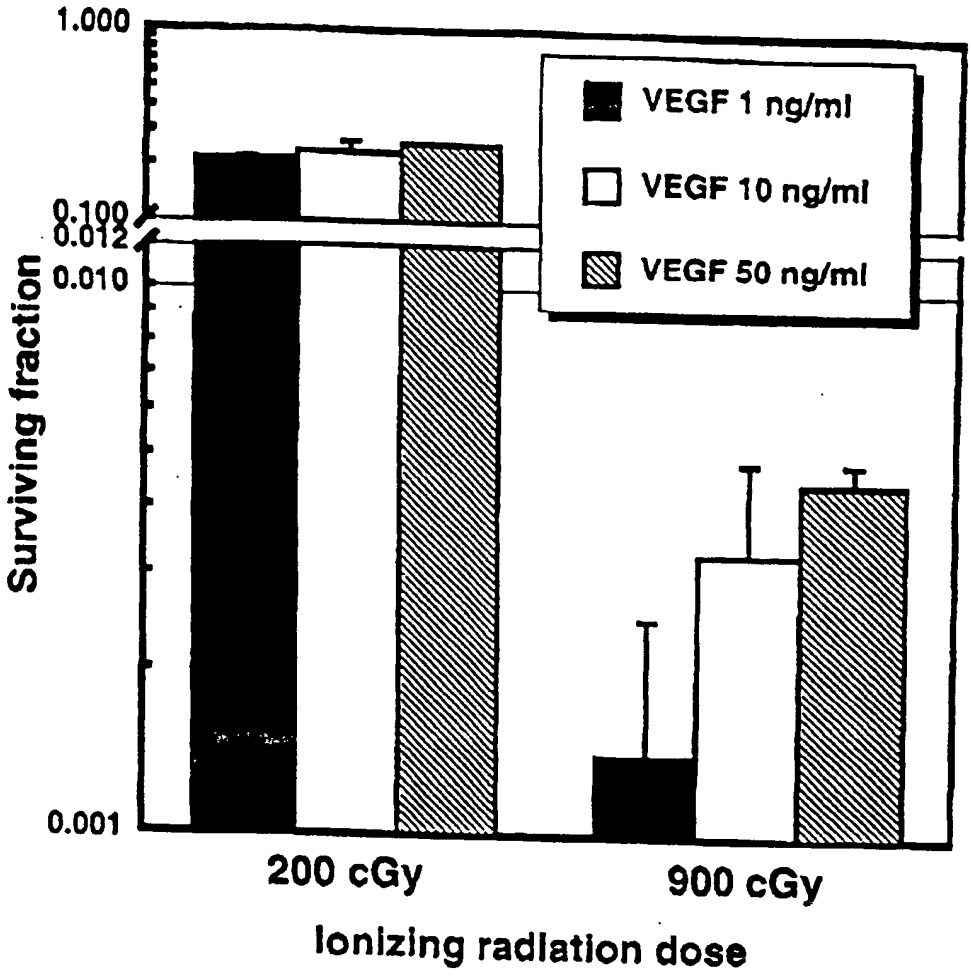
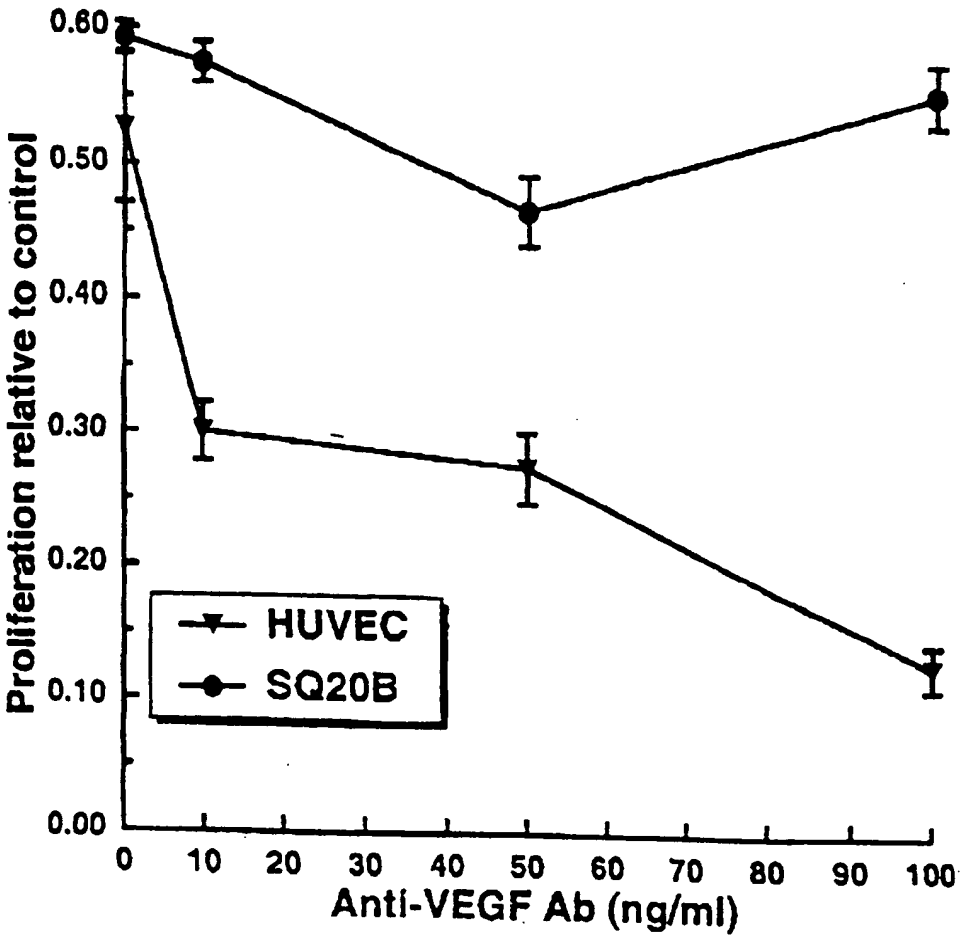

Proliferation relative to control
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
35
40
45
50
VEGF (ng/ml)

FIGURE 4B



PLACEHOLDER—REPEAT EXPERIMENT PENDING

FIGURE 4C


0.60
0.50
0.40
0.30
0.20
0.10
0.00
Proliferation relative to control
HUVEC
SQ20B
0
10
20
30
40
50
60
70
80
90
100
Anti-VEGF Ab (ng/ml)

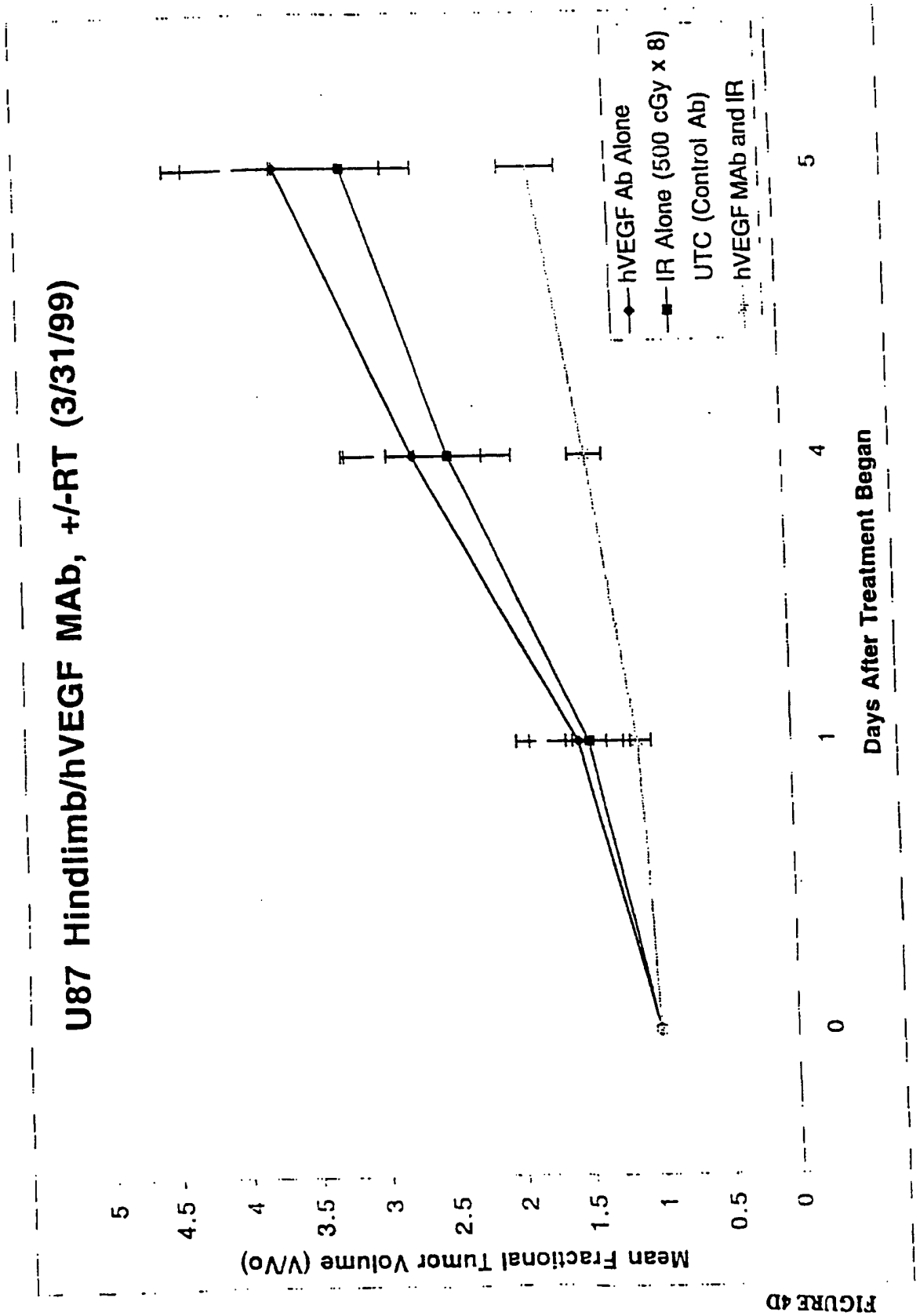

U87 Hindlimb/hVEGF MAb, +/-RT (3/31/99)
Mean Fractional Tumor Volume (V/Vo)
Days After Treatment Began
hVEGF Ab Alone
IR Alone (500 cGy x 8)
UTC (Control Ab)
hVEGF MAb and IR
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
1
4
5

FIGURE 4D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09255

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 39/395; C07K 16/00
US CL :424/130.1; 530/387.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, WEST
search terms: VEGF, antibodies, inhibitors, radation, chemotherapy, cancer.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KATOH et al. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inihibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. Cancer Research. 01 December 1995, Vol 55, No. 23, pages 5687-5692. See entire document.	1-2
Y	KATOH et al. Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. Cancer Research. 01 December 1998, Vol. 58, No. 23, pages 5565-5569. See entire document.	1-2

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search
29 JULY 2000

Date of mailing of the international search report
14 AUG 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/09255

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BORGSTROM et al. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endotheial growth factor neutralizing antibody: Novel comcepts of angiostatic therapy from intravital videomicroscopy. Cancer Research. 01 September 1996, Vol. 56, pages 4032-4039. See entire document.	1-2
Y	GOLDMAN et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proc. Natl. Acad. Sci. USA. July 1998, Vol. 95, pages 8795-8800.	1